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14. ABSTRACT The focus of this grant is to investigate immunoprofiles for serum antibodies to aberrant glycans in human and animal models of mesothelioma. This is accomplished using a one of a kind printed glycan array which is at NYU School of Medicine (NYUSoM). It is hoped that these experiments will allow us to diagnose and prognosticate mesothelioma more accurately in the future. We have been severely limited by our ability to start the human mesothelioma glycoprofiles as well as the animal profiles due to delivery and set up times for our one of a kind glycomics laboratory at NYUSoM. We summarize the situation in the progress report with the good news that we will be moving onwards in June with these studies.			
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## INTRODUCTION

The overall goal of our investigations is to identify a serum anti-glycan antibodies (AGAs)-based immunosignature of human malignant mesothelioma (MM) that would allow for identification of individuals, including military personnel, at high-risk for MM due to their potential long-term exposure to a carcinogenic form of asbestos, in time for an effective early intervention. Since such an immunosignature and the accompanying serum AGA immunoprofile reflect overall health, and more specifically immune health status of a person, both parameters are likely to also provide an insight into biological factors contributing to a susceptibility to this malignancy.

This project is funded in order to investigate immunoprofiles of serum anti-glycan antibodies recognizing Mesothelioma-derived aberrant glycans in human subjects and in animal models of Mesothelioma. This is accomplished using a one of a kind printed glycan array (PGA), which was developed by us at the New York University School of Medicine (NYU SoM), and was expanded by an addition of 182 novel glycan probes, many of which are Mesothelioma-specific.

Here we report:

- (i) the preliminary results from immunoprofiling serum specimens collected monthly throughout the entire course of this 14-months experiment as proposed in the **Specific Aim II A** of this project; and
- (ii) an outline and preliminary results of the experiment proposed in the **Specific Aim II B**.

## BODY

**Specific Aim II A: Using a rat model of asbestos-induced MM, immunoprofile serum AGAs using PGA and define temporal changes in this immunoprofile as mesothelial carcinogenesis develops and progresses during the 13-month experiment.** The study was carried out using three groups of Fischer 344 female rats: (i) 32 animals exposed to intraperitoneal (IP)-applied asbestos/crocidolite as MM-inducing agent; (ii) 32 animals exposed to silica fiber as an IP-applied control for asbestos/crocidolite exposure, and (iii) 8 animals, for a one-time sham saline IP injection – as a control for age-related changes in AGA immunoprofiles.

In the previous report, we presented a description of this longitudinal experiment and a figure showing the changes in weight over time for all animal groups. Inter-experimental follow-up procedures included (i) daily observations by the veterinarian staff, (ii) twice-weekly observations by the research associates participating in the project, and (iii) monthly weight measurements and blood collection. At the experimental end-point, animals were sacrificed according to the recommendations of IACUC. If animals exhibited symptoms of ill health, stress or fatigue prior to the study's endpoint, they were euthanized and necropsied before the study end-point at the "humane point".

End-point necropsy procedures included: detailed observations of the internal organs of each individual experimental animal, including photographic records of selected cases, and the collection of tumors and other tissues, including serum from each experimental animal. All observations made during the necropsy procedures were recorded and later transcribed into a full report, which is presented as **Attachment 1**. This document will also be included as an "Additional Dataset" in the report of this study, which is currently under preparation for publication.

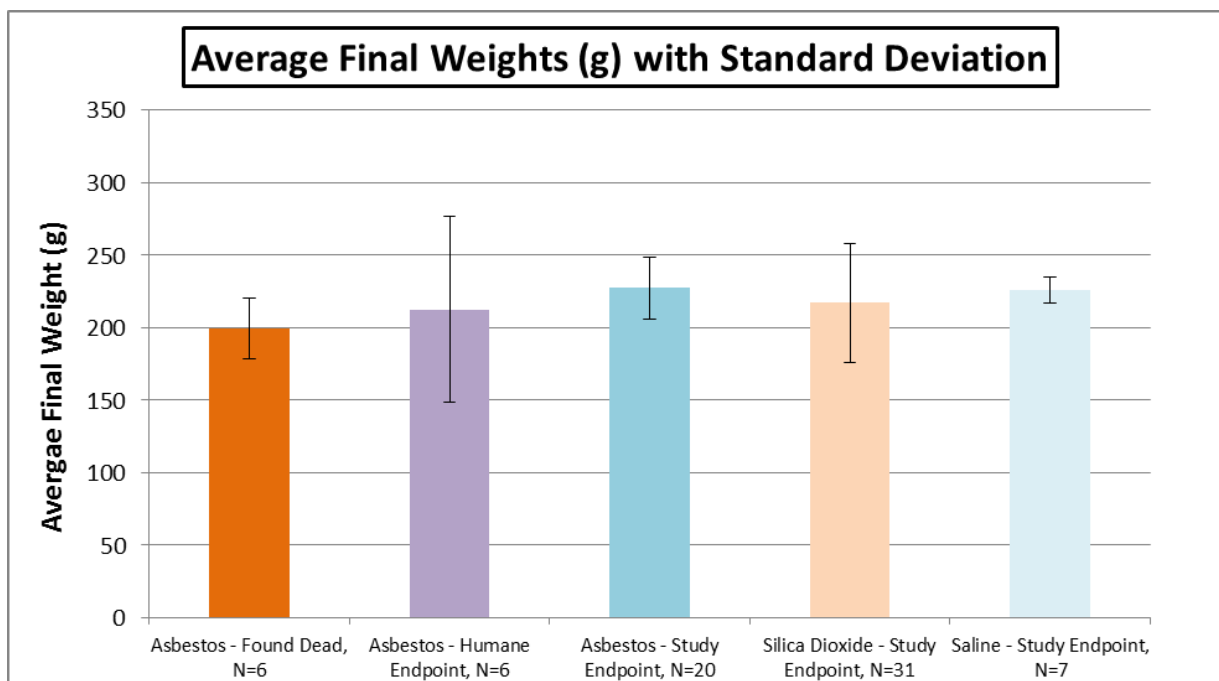
### **Discussion of the preliminary findings:**

1. There were variable responses to the peritoneally injected asbestos among the 32 experimental animals: 25 animals developed mesothelioma (78%), and 7 animals were found disease-free at the end-point necropsy.

Among the 25 animals that developed mesothelioma, 13 animals reached the study end-point: 7 animals had fully developed peritoneal mesothelioma, and 6 animals had minimal disease in the form of miliary tumors. Twelve animals did not reach study end-point: 6 animals were found dead of the disease and 6 were euthanized at the later stages of the experiment due to the animals' rapidly deteriorating health, which was the result of quickly progressing mesothelioma. This observation is very significant since it implies distinctly different individual responses to a carcinogen, similar to humans.

These different biological responses to a carcinogen are also indirectly manifested as differences in the weights of individual animals. In extreme cases, such differences are the result of "wasting" or the accumulation of large volume of ascites. This is reflected by large standard deviations in average final weight as shown in **Figure 1**, particularly in the group of asbestos-injected animals which had to be euthanized at the humane end-point.

**Figure 1: Distribution of the body weight in the experimental animal groups at the study end-point or at the time of animal death due to the asbestos-induced mesothelioma progression. Based on the study outcome, asbestos-injected animals are further separated into the sub-groups of "Found dead of the disease, N=6", "Humane Endpoint, N=6" and "Study Endpoint, N=20".**



2. During the experimental end-point necropsies out of 32 animals injected with silica, 31 animals were found disease-free and one animal was found to have developed a sarcoma tumor.

3. All control saline-injected animals were found disease-free at the experimental end-point necropsies, and control saline-injected animals and silica-injected animals survived until experimental end-point.

Preliminary results of rat serum immunoprofiling are presented in Attachments 2 and 3.

**Attachment 2** shows three sets of bar-graphs presenting fluorescence intensities of rat serum anti-glycan antibodies binding to glycan probes present in our Printed Glycan Array NYU PGA-400. The top bar-graph shows pre-injection AGAs for all three experimental animal groups:

“asbestos”, “silica” and “saline”. In all three bar graphs, asbestos-injected animals are colored red, silica-injected animals are colored blue, and saline-injected animals are colored green. The middle bar-graph shows one-month post-injection AGAs for all three experimental groups, and the bottom bar-graphs shows AGAs at the study endpoint for all three experimental groups. The endpoint AGA immunoprofiles are obtained from the sera of individual animals, whereas the AGA “pre-injection” and “1 month post—injection” immunoprofiles are obtained from the pooled sera of three to five animals. Sera of these animals were pooled due to the low volume of blood collected from the tail vein of animals still young and small at early experimental time-points.

The bar-graphs have been aligned in a way that allows us to observe changes in the individual AGAs over time between the experimental animal groups. For instance, appearances of specific AGAs in response to the asbestos injection are detectable in the “1 month post-injection” immunoprofiles. Significant and distinct differences between serum AGAs intensities in “asbestos” vs. “silica” vs. “saline” rats at the study endpoint are also immediately noticeable.

**Attachment 3** presents structures of selected glycans exhibiting distinct serum AGA binding patterns in “asbestos” vs. “silica” rats four weeks following injections. Antibodies against glycans marked by “X” show significant dynamics in response to asbestos exposure and during mesothelioma development in both rat and human populations.

We are preparing the results of this experiment for publication, which will be submitted once we complete rat serum immunoprofiling and analyses. This manuscript will be included in the final study report.

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**Specific Aim II B: Use the syngeneic II-45 cell line xenograft in rat model of asbestos-induced mesothelioma to correlate mesothelioma tumor growth with rat serum anti-glycan antibodies (AGA).**

The goals of this experiment are: (i) to identify glycans showing the dynamics of anti-glycan antibodies during outgrowth of syngeneic mesothelioma tumors, implanted intraperitoneally (IP) or subcutaneously (SC), by comparing immunoprofiles of saline-injected control rats to rats with the implanted tumor cells, and (ii) to identify glycans showing the dynamics of anti-glycan antibodies in response to the chemotherapy drug Gemzar by comparing immunoprofiles of Gemzar-injected rats with the immunoprofiles of saline-injected control rats. Gemzar is an anti-cancer drug often used in MM treatment, and is known to have immunomodulatory effects. In this experiment we investigated whether this immunomodulatory effect is detectable on the level of the AGA dynamics in healthy animals.

Preparations for this study have been described in the previous report. Briefly, we have re-grown fresh stocks of syngeneic rat mesothelioma II-45 cells and performed testing for a panel of animal pathogens, with specific focus on rat pathogens. As determined by Charles River Research Animal Diagnostic Services, our II-45 cell line was pathogen-free, and was ready for injections as proposed in the second arm of the study.

Experimental Design and Schedule are shown in **Table I** below.

**Table I: Schedule of an experiment performed under Specific Aim II B:**

Group		6/13/14	6/27/14	7/1/14	7/11/14	7/15/14	7/24/14	7/25/14	7/29/14	7/31/14
		Day - 18	Day - 4	Day 0	Day 13	Day 14	Day 23	Day 24	Day 28	Day 30
Control	6 IP Females	Bleed	Bleed	Injection	Sacrifice: bleed	Bleed	Sacrifice: bleed	Bleed	Sacrifice: bleed	Sacrifice: bleed
	6 SC Females	Bleed	Bleed	Injection	Sacrifice: bleed	Bleed	Sacrifice: bleed	Bleed	Sacrifice: bleed	Sacrifice: bleed
II-45 Cell Lines	12 IP Females	Bleed	Bleed	Injection	Sacrifice: bleed & harvest tumors	Bleed	Sacrifice: bleed & harvest tumors	Bleed	Sacrifice: bleed & harvest tumors	NA
	12 SC Females	Bleed	Bleed	Injection	Sacrifice: bleed & harvest tumors	Bleed	Sacrifice: bleed & harvest tumors	Bleed	Sacrifice: bleed & harvest tumors	NA
Gemzar	12 TV Females	Bleed	Bleed	Injection	Sacrifice: bleed	Bleed	Sacrifice: bleed	Bleed	Sacrifice: bleed	Sacrifice: bleed

Prior to the first blood draw, 3-4 week old Fischer F344 female rats with tattooed tails featuring unique identifying numbers were acclimatized for 18 days. After 3 weeks of acclimatization, the experiment began. In accordance with NYUSM DLAR blood drawing policy, blood draws were performed by tail nicking in order to obtain 200-500  $\mu$ L of blood per draw.

On day 0,  $1 \times 10^6$  syngeneic rat mesothelioma cells were injected into the rats' dorsal flanks in 0.2 mL of HBSS via a subcutaneous (N=12) or intraperitoneal (N=12) injection. Control animals were injected with 0.2 mL of HBSS via a subcutaneous (N=6) or intraperitoneal (N=6) injection. A 0.2 mL solution of Gemzar (40 mg/kg body weight) in HBSS was injected into "Gemzar" rats (N=12) via a tail vein.

Animals were observed daily by DLAR staff and at least two times per week by research associates participating in the project. Tumor growth was monitored over a 4 week period using a digital caliper.

To characterize an "early" stage of tumor growth, on day 13 tumors were removed from euthanized animals in the subcutaneous (SC) and intraperitoneal (IP) cell line group. Resected tumors were examined for their pathological features, and stored in formalin for further analysis.

For comparison, 6 animals from the subcutaneous (SC) and intraperitoneal (IP) control group, and 3 animals from the Gemzar group were also sacrificed on day 13. All animals were

sacrificed according to the recommendations of IACUC. At day 24 and day 28, tumors from the subcutaneous (SC) group and intraperitoneal (IP) cell line group were harvested and stored in a similar manner. The remaining control and Gemzar animals were sacrificed on day 30 at the conclusion of the experiment.

End-point necropsy procedures included: detailed observations of the internal organs of each individual experimental animal, including photographic records of selected cases, and the collection of tumors and other tissues, including serum from each experimental animal. All observations made during the necropsy procedures were recorded and later transcribed into a full report, which is presented as **Attachment 4**. This document will also be included as an “Additional Dataset” in the published report of this study.

AGA immunoprofiling of serum specimens is in progress.

### **Classification of rats implanted with mesothelioma syngeneic cells, N=24, based on the study outcome:**

Disease free, study endpoint, N=0;

- Minimal disease, study endpoint, N=0;
- Fully developed disease, study endpoint, N=13;
  - SC: N=9;
  - IP: N=4;
- Fully developed disease, humane endpoint, N=7;
  - SC: N=3;
  - IP: N=4;
- Fully developed disease, found dead, N=4;
  - IP: N=4;
    - Found dead on 7/23/14, N=3;
    - Found dead on 7/28/14, N=1;

### **Main Observations from the Specific Aim II B animal experiment:**

- Saline-injected rats: no health problems observed, no tumors found at the necropsy.
- Unusually fast growth of implanted Mesothelioma cultured cells noted after the death of:
  - 3 IP rats on day 22 of the study;
  - 1 IP rat on day 27 of study;
- Due to faster than expected growth of implanted Mesothelioma cultured cells, the experiment concluded faster than expected.

## **KEY RESEARCH ACCOMPLISHMENTS**

1. We have further expanded the array platform, NYU-PGA-400 by adding 182 novel glycan probes, many of which are human Mesothelioma-specific. It is expected that this expanded glycochip will allow us to better diagnose and prognosticate Mesothelioma earlier during its development, and obtain larger volume of information about the pathobiology of the immune system under a pressure of the asbestos exposure and during the mesothelioma development.

2. We have generated a large library of rat anti-glycan immunoprofiles immunoprofiles obtained from the AGA immunoprofiling of sera collected periodically in the 13-month experiment following exposure of animals to asbestos – as a carcinogen, or to silica – as an irritant. This library of AGA longitudinal immunoprofiles is already showing a great value as a source of information allowing an insight into the (rat) immune system responses and into the AGA dynamics following exposure to environmental factors harmful also to the human health.



3. We have identified a set of glycans indicated by a dynamics of the immune response to asbestos in both rats and humans. Identification of the real targets of antibodies recognizing these glycans should help to develop the mesothelioma-preventive strategies. Such preventive strategies may include immune system-correcting / reconstituting intervention, as a single modality or as a companion-preventive therapeutics.

## **REPORTABLE OUTCOMES**

Results presented here in all four attachments are components of publications currently under preparation. These publication(s) will be submitted once we complete rat serum immunoprofiling and analyses of both animal experiments. These manuscripts will be included in the final study report.

## **CONCLUSIONS**

1. Experimental rats show distinctly different individual responses to both asbestos-environmental carcinogen, and silica – environmental irritant. This observation validates one of our initial working hypotheses and is very significant since it implies different individual immune responses to carcinogen(s) and irritant(s), similar to humans. Furthermore, this observation also confirms a suitability of Fisher 344 rat as a model-animal to explore mesothelioma-preventive intervention strategies.

2. Certain anti-glycan antibodies are induced or show marked dynamics of their concentration in circulation in response to the bolus - peritoneal injections of asbestos or silica; this dynamics of the “early response AGAs” is observed in the first serum collected four weeks following injections, it is more pronounced in some experimental animals than in the others, and it differs between asbestos-injected and silica-injected animals.

3. The dynamics of certain AGAs over time appears to correlate with the type of animal response to asbestos exposure: this is a preliminary observation - the final conclusion will be reached after completion of immunoprofiling of all longitudinally collected sera of “asbestos rats”.

4. It will be important to resolve the time-period of four weeks following injections of asbestos and silica, to capture earliest points of the “early response AGAs” generation and the presence in the circulation of exposed animals. It will also be important to determine an isotype of the “early response AGAs” and “response AGAs” to better understand immune system components and mechanisms involved in the dynamics of the response to asbestos exposure.

5. Serum antibodies against selected set of glycans show significant dynamics in response to asbestos exposure, and during mesothelioma development in both rats and humans. Identification of the real targets of antibodies recognizing these glycans should help to develop the mesothelioma-preventive strategies. Such preventive strategies may include immune system-correcting / reconstituting intervention, as a single modality or as a companion-preventive therapeutics.

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**APPENDICES:**

**Attachment 1:** Transcripts of the observations made during the necropsies in all three experimental groups of animals intraperitoneally (IP)-injected with asbestos/crocidolite, animals IP-injected with silica fiber, and animals sham-IP-injected with saline.

**Attachment 2:** Three sets of bar-graphs presenting fluorescence intensities of rat serum anti-glycan antibodies in all three experimental groups. The bar-graphs show AGAs in sera collected “pre-injection”, “one-month post-injection” and at the “study endpoint”.

**Attachment 3:** Structures of selected glycans exhibiting distinct serum AGA binding patterns in “asbestos” vs. “silica” rats four weeks following injections. Antibodies against glycans marked by “X” show significant dynamics in response to asbestos exposure and during mesothelioma development in both rat and human populations.

**Attachment 4:** Experimental schedule and notes from observations made during the necropsies of all groups of animals injected with the syngeneic cell line xenograft, saline, and Gemzar.